

## Lipids stimulate spore germination in the entomopathogenic ascomycete *Ascosphaera aggregata*

R.R. James<sup>1</sup> & J.S. Buckner<sup>2</sup>

<sup>1</sup>USDA-ARS Bee Biology and Systematics Laboratory, Department of Biology, UMC 5310, Utah State University, Logan, UT 84322-5310, USA; <sup>2</sup>USDA-ARS Red River Valley Agricultural Research Center, Biosciences Research Laboratory, P.O. Box 5674, Fargo, ND 58105-5674, USA

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### Abstract

The alfalfa leafcutting bee (*Megachile rotundata*) is solitary and managed on a large scale for pollination of alfalfa seed crops. The bees nest in holes drilled in wood or polystyrene blocks, and their larvae are highly prone to a fungal disease called chalkbrood. The most prevalent form of chalkbrood is caused by *Ascosphaera aggregata*, but this ascomycete is difficult to culture. Hyphae will grow on standard fungal media, but spore germination is difficult to achieve and highly variable. We found that germination can be enhanced with oils. Lipids derived from plants and bee larvae increased germination from 50% (without oil) to 75–85% (with oil). Percent germination was significantly greater in the presence of lipids but germination was not significantly different when different oils, including mineral oil, were used. *A. aggregata* spores oriented along the oil–aqueous interface in the broth in a polar fashion, with swelling and germ tube formation always occurring into the aqueous portion of the broth. The other half of the spore tended to attach to a lipid droplet, where it remained, without swelling, during germ tube formation. The physical attachment of spores to the oil–aqueous interface is what most probably stimulates spore germination, as opposed to some nutritional stimulation. However, further research is needed to determine if and where the spores encounter such an interface when germinating in the host gut, where germination normally occurs.

**Key words:** alfalfa leafcutting bee, chalkbrood, fatty acids, lipids, *Megachile rotundata*, spore germination

### Introduction

The alfalfa leafcutting bee, *Megachile rotundata*, is managed for pollination of alfalfa seed. Chalkbrood is a disease of *M. rotundata* larvae, caused by the fungus *Ascosphaera aggregata*, and causes economic losses in the US because it prevents alfalfa seed producers from being able to maintain bee populations from one year to the next. Chalkbrood is initiated when *A. aggregata* spores are consumed by a larva and germinate in the gut [1, 2]. The fungus then grows in all the tissues of the developing bee larva, eventually killing it. After host death, ascomata (spore cysts) form underneath the cuticle of the dead larva, and

distinct spore balls form within the ascomata [1, 2]. The age of the host at which infection most commonly occurs is not clear, but most sporulating cadavers are fully formed, late-instar larvae that die just prior to cocoon formation [3].

The alfalfa leafcutting bee is a solitary bee, and females make individual nest cells in holes found in wood. The cells are composed of pieces of leaves or petals they cut from live plants, and are composed in a series along the length of the hole. A nesting female provisions each cell with pollen and nectar before laying an egg. Most commonly, the insect is univoltine, and the larvae overwinter in the prepupal stage. A portion of the population may become multivoltine, with new adults emerging mid-season.

The frequency of multivoltinism is more common in climates with longer summer periods. Chalkbrood mortality occurs during the summer, and the spores overwinter on the sporulating cadavers. When healthy adults emerge from their cells, they become contaminated with spores from their infected siblings [4], perpetuating the spread of the disease.

Twenty-one species of *Ascosphaera* have been identified and described to date [5, 6], all of them associated with bees, either as pathogens or as saprophytes that grow in the pollen provisions provided by the mother bee. Of these species, *A. aggregata* is the most common in *M. rotundata* in the US, and is one of the most difficult to culture. Spore germination rates on artificial media are low and variable [7–9].

Youssef [10] developed an agar-based medium for culturing *A. aggregata* that, for the first time, provided spore production. One ingredient in the medium was canola shortening. Youssef [10] speculated that fatty acids in the shortening served as a carbon source for the developing fungus and triggered maturation. However, in addition to spore production, the medium also provided for very high germination rates. Due to these results, we decided to test whether lipid can enhance spore germination. Here, we compare the effects of different plant lipids, lipid extracts from mature bee larvae, and mineral oil.

## Materials and methods

### *Source of fungal spores*

Sporulating cadavers of alfalfa leafcutting bee larvae were collected in May from nesting blocks located outside the laboratory in North Logan, Utah, and stored at 4 °C until needed (2–3 months).

### *Culture medium*

The base medium we used was a modified version of the V-8 juice plug agar developed by Youssef [10], and included the following (per 100 ml): 50 ml filtered low sodium V-8 Juice<sup>®1</sup> (Campbell Soup Co., Camden, NJ), 5.3 g maltose, 1.6 g yeast

extract, 0.11 g MgSO<sub>4</sub>, 0.04 g thiamine, 1.1 mg biotin, and 7.5 ml Graces Medium (Invitrogen Co., Grand Island, NY). Deionized water was used to bring the broth to volume. The pH was adjusted to 6.0. We refer to this medium as V-8 broth.

### *Assessing lipid content of culture broth and pollen provisions*

#### *Culture broth*

We expected the V-8 both to have a very low lipid content. None of the ingredients added to the broth contained lipids except perhaps the V-8 juice. The label for V-8 juice says it contains no fats; however, we suspected that it must contain some vegetable lipid, even if in only a very small quantity.

We extracted all lipids from 40 ml of V-8 broth using a chloroform-methanol extraction. Broth was placed in a beaker with a 2:1 mixture of CHCl<sub>3</sub>:MeOH, using an equal volume of CHCl<sub>3</sub>:MeOH and broth. The beaker was placed in a sonicating bath for approximately 1.5 h, and then was transferred to a separatory funnel and vigorously mixed with deionized water (using 0.2 ml of water per ml of broth). This mixture was then allowed to separate, and the water layer was extracted again with additional CHCl<sub>3</sub>. The combined CHCl<sub>3</sub> layers were backwashed with water, and the solvents were evaporated under nitrogen.

The V-8 broth lipid extract was fractionated into triacylglycerols (TAGs) and free fatty acids (FFAs) by silica column chromatography. The TAG fraction was hydrolyzed, and the recovered fatty acids converted to methyl esters (FAME) for CGC-MS analysis. The FFA fraction was also analyzed by CGC-MS as FAME.

For thin layer chromatography (TLC) analysis, the residue was dissolved in 840 µl CHCl<sub>3</sub>, and 4 µl (1/210) was spotted onto a HPTLC plate and developed in HEF (80:20:1). The sample was spotted along with standards.

For silica column fractionation, a silica column was prepared (0.183 mg). The residue was thoroughly suspended in 2 ml hexane using sonication and heating. We added this suspension to the column, and collected Fraction 1a by rinsing the column with ~8 ml hexane. Fractions 1b, 2–4 were collected by sequential elution with hexane/*t*-BME (99.5:0.5), hexane/*t*-BME (96:4), hexane/acetic acid (100:2) and *t*-BME/acetic acid (100:0.2). The

<sup>1</sup> This article reports the results of research only. The mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

solvents were evaporated and the residues were stored at  $-20^{\circ}\text{C}$  under argon.

The Fraction 2 residue was transferred to a 1-ml Reacti-Vial with  $\text{CHCl}_3$ , then we added  $40\ \mu\text{l}$  benzene followed by  $760\ \mu\text{l}$  of 5% KOH in MeOH. The vial was capped and the reaction mixture was heated to  $70^{\circ}\text{C}$  and held for 3 h. After cooling, the reaction mixture was transferred to a 30-ml separatory funnel, neutralized with  $7\ \mu\text{l}$  of 1.0 M HCl and partitioned with  $\text{CHCl}_3$ . The water layer was extracted with additional  $\text{CHCl}_3$  and the combined  $\text{CHCl}_3$  backwashed with water.

The hydrolyzed samples from Fractions 3 and 4 were each transferred into a 1-ml Reacti-Vial with  $\text{CHCl}_3$ ,  $40\ \mu\text{l}$  benzene were added followed by  $760\ \mu\text{l}$  of  $\sim 10\%$  HCl in MeOH. The reaction mixtures were heated to  $70^{\circ}\text{C}$  and held for 1 h. After extraction  $2\times$  with  $\text{CHCl}_3$ , the FAME samples were transferred into  $300\ \mu\text{l}$  insert vials (ready for CGC-FID analysis). Also transferred into a  $300\ \mu\text{l}$  insert vial for CGC analysis was Fraction 1b.

Three V-8 broth fractions were analyzed by CGC-MS: (1) Fraction 1b (wax esters), (2) Fraction 2 (TAG-fatty acids as FAME after KOH and HCl/MeOH), and (3) Fraction 3 (FFAs as FAME after HCl/MeOH).

#### *Pollen provision*

Lipids from digested pollen provision are very likely to be released into the insect gut and thus become available to spores, and for this reason, we wanted to know the composition of lipids in the provision. It may have been preferable to test actual gut contents, but due to the small size of the insects, we were unable to develop a method to extract a sufficient amount of material for analysis, nor could we extract the gut contents and be confident that we were not contaminating the sample with insect fluids. The lipid composition of pollen provisions should approximate the lipid composition of the gut because the larval gut becomes filled entirely with pollen provision.

Over the course of the summer, a large number of provisions (several hundred) were collected from new cells of the alfalfa leafcutting bee (within a day or two of being formed) and stored together at  $4^{\circ}\text{C}$ . The bee cells were collected from an alfalfa seed farm near Tremonton, UT. This large sample was mixed together, and then a small portion was sub-sampled ( $0.91\ \text{g}$ ), placed in a 20-

ml glass vial, chilled to  $-80^{\circ}\text{C}$ , and then lyophilized overnight. After lyophilization, the provision was weighed again to determine the amount of free water. The lipids were extracted by sonicating the lyophilized provision with  $\text{CHCl}_3/\text{MeOH}$  (2:1) in a water bath, and then were recovered by removing the  $\text{CHCl}_3$  layer and evaporating off the solvent.

Neutral lipids were analyzed by HPTLC using a silica gel plate and HEF (80:20:1). A 1/10 ( $300\ \mu\text{l}/3000\ \mu\text{l}$  in hexane) was removed from the sample ( $4.35\ \text{mg}$ ) and applied to a silica gel column ( $0.183\ \text{g}$  in a small transfer pipette). Not all of the residue dissolved in  $3000\ \mu\text{l}$  of warmed hexane, but the suspension was thoroughly mixed and  $300\ \mu\text{l}$  quickly removed with a  $500\text{-}\mu\text{l}$  syringe and applied to the column. The column was eluted as follows: Fraction 1a =  $\sim 8\ \text{ml}$  of hexane, Fraction 1b =  $8\ \text{ml}$  hexane/*t*-BME (99.5:0.5), Fraction 2 =  $8\ \text{ml}$  hexane/*t*-BME (96:4), Fraction 3 =  $8\ \text{ml}$  hexane/acetic acid (100:2), Fraction 4 =  $8\ \text{ml}$  *t*-BME/acetic acid (100:0.2), and the Phospholipid Fraction =  $8\ \text{ml}$  MeOH/*t*-BME/ammonium acetate (5:4:1).

For Fractions 1a–4,  $500\ \mu\text{l}$   $\text{CHCl}_3$  was added, and then  $5\ \mu\text{l}$  was spotted on an HPTLC silica gel plate and developed in HEF (80:20:1). Fraction 2 was plated across a  $10 \times 10\ \text{cm}$  silica gel HPTLC plate and developed in HEF (80:20:1). Two bands were visible on a light box and were outlined with a pencil. Each band was scraped with a razor blade and transferred into a glass column. Lipid was eluted from the silica gel with  $\text{CHCl}_3$ . The upper band was presumed to be TAG and was stored away. One-third of the lower band (presumably FFA) was transferred into a 1-ml Reacti-Vial.

Benzene ( $40\ \mu\text{l}$ ) and 10% HCl in MeOH ( $760\ \mu\text{l}$ ) was added to the 1/3 portion of lower band residue. The reaction mixture was heated at  $70^{\circ}\text{C}$  for just 10 min. After cooling the vial, the reaction mixture was transferred into a 30-ml separatory funnel and the vial rinsed with  $\text{CHCl}_3$ . Water was added to the reaction mixture plus  $\text{CHCl}_3$ , and then the  $\text{CHCl}_3$  layer was removed after shaking (partitioning) and the water layer re-extracted with  $\text{CHCl}_3$ . The combined  $\text{CHCl}_3$  was backwashed with water. The HCl/MeOH reacted lower band was analyzed by HPTLC. Five microliters of  $150\ \mu\text{l}$  in  $\text{CHCl}_3$  was spotted on a plate along with  $5\ \mu\text{l}$  of  $300\ \mu\text{l}$  of un-reacted lower band and a wax ester standard. The reacted 1/3

sample was dissolved in 500  $\mu$ l  $\text{CHCl}_3$ , and 1  $\mu$ l was injected onto a CGC-MS.

We analyzed the fatty acids from the provision TAG fraction and compared the data with the results of our analysis of the FFA fraction.

#### *Extraction of bee lipids*

Healthy bee larvae from Canada were purchased in February from JWM Leafcutters, Inc. (Nampa, ID) as overwintering 5th instars and stored at 4 °C until needed (5–6 months). Bee larvae were removed from the nesting cells, and 20–200 bees were used per extraction. The larvae were rinsed in hexane for about 4 min to remove cuticular lipids, immediately dipped for 30 s in chloroform ( $\text{CHCl}_3$ ) and then placed in a beaker with a 2:1 mixture of  $\text{CHCl}_3$ :MeOH, using 1 ml of mixture per bee. The beaker was placed in a sonicating bath for approximately 1.5 h, until all the bees sank to the bottom of the beaker. The mixture of solvents and bees was then placed in a porcelain mortar and homogenized. The homogenate was returned to the beaker and sonicated again for 45 min before being transferred to a separatory funnel and vigorously mixed with deionized water (0.2 ml water per bee). This mixture was then allowed to separate, and the water layer was extracted again with additional  $\text{CHCl}_3$ . The combined  $\text{CHCl}_3$  layers were backwashed with water, and the solvents were evaporated under nitrogen.

#### *Effect of oils on spore germination*

##### *Experiment 1: Canola oil and bee lipids*

To determine whether a plant- or insect-derived lipid would increase spore germination, we incubated *A. aggregata* spores in three different media: (1) V-8 broth with no added lipid (as a control), (2) broth with 1% canola oil (Crisco®, Proctor & Gamble, Cincinnati, OH), and (3) broth with 1% bee lipid extract. Lipids were added to the broth by first mixing the oil with an equal volume of 0.1% Triton X-100 (ICN Biomedicals, Inc., Aurora, OH) on a vortex mixer until an emulsion was formed, and this emulsion was added to the broth, and then the broth was autoclaved at 121 °C for 20 min. The control had Triton X-100 added at the same rate as the lipid treatments (final concentration was 0.001%).

For each experimental run (replicate block), spores were scraped from a single cadaver in a 1 ml sterile plastic microcentrifuge tube, and then ground gently using a plastic pestle to break apart the spore balls. The spores were mixed with 1 ml of sterile deionized water, using a vortex mixer on the highest setting for 2 min, to further break apart the spores. The large particles in the suspension were allowed to settle for 20 min, and then a sample of approximately 0.1 ml was taken from the middle of the suspension using a pipette. This sample was diluted appropriately (usually about 10-fold) to obtain a concentration of  $2.0 \times 10^7$  spores per ml. A haemocytometer was used to measure spore concentration.

We mixed 0.9 ml of each medium with 0.1 ml of spore suspension in the wells of a 24-well plate to obtain a final concentration of  $2.0 \times 10^6$  spores/ml. The plates were incubated on a rotary titer-plate shaker (Lab-Line Instruments Inc., Melrose Park, IL) in a  $\text{CO}_2$  incubator (30 °C and 20%  $\text{CO}_2$ ), in the dark. The shaker was set to the fastest speed possible without spilling any broth (set at 3.2 which is approximately 350 rpm with a 0.3 cm orbit). After 24 h of incubation, we checked spore germination by placing 10–20  $\mu$ l of sample on a glass slide and examined it using phase contrast microscopy at 400–600 $\times$  magnification. In the control broth, the slide was visually scanned in a systematic fashion, and all the spores in each field of view were evaluated for germination until the number of spores that had been examined reached 200. *A. aggregata* spores swell at one end before germination, but spores were not considered to have germinated unless a distinct germ tube had formed from the swollen end.

For the lipid treatments, we noticed that the spores tended to attach to the lipid droplets in the broth, and the attached spores seemed much more likely to be germinated than those that were detached. Thus, the distribution of germinated spores on the prepared slides was not random, making it difficult to take a true random sample. To quantify whether attached spores were really more likely to be attached to lipid droplets, the slide was visually scanned in a systematic fashion, but we separated the counts for spores that were attached to lipid droplets and those that were not. We counted 200 spores in each category. The majority of spores were attached to lipid droplets, so a considerably larger area on each slide had to

be scanned in order to obtain 200 detached spores. Percent germination was then determined for each category separately.

The experiment was replicated in a random block design. From past experience, we noticed that sporulating cadavers can vary significantly in spore germinability; therefore, the experiment was set up as a block design, using the spores from each bee cadaver as a block (i.e., all treatments in a replicate used spores from the same cadaver), with nine replicate blocks.

ANOVA on an arcsine-square-root transformation of the proportion of spores germinated was used to determine if the effects of lipids were significant. A Duncan's LSD test was used to compare percent germination for spores in the control and spores either attached to or detached from a lipid drop, in each of the lipid treatments.

#### *Experiment 2: Biologically derived oils*

In this test, we added a second plant oil, linseed (Aldrich Chemical Co., Milwaukee, WI), in addition to canola oil and the bee lipid extracts. The purpose was to determine if fatty acid composition had an effect on spore germination. We selected three oils that had very different fatty acid compositions (Table 1), and these oils were tested at two different concentrations. In addition, we used a slightly different sampling technique that allowed us to take a more random sample of spores than before. We wanted a truly random sample to determine whether the lipids were actually enhancing germination, as opposed to the spores just being more likely to attach to the lipids after germination.

V-8 broth was prepared as described above, and then autoclaved and dispensed into the wells of a 24-well plate. Sterile oils (without surfactant)

were then added to each well and emulsified in the broth using a sonicator. All the oils were autoclaved to sterilize them, except for the bee lipid extract. The chloroform-methanol extraction procedure produced a sterile lipid product, so no further sterilization was required. The lipids were tested at concentrations of 1 and 2%. Spores were added to the media as described above, and incubated in the dark on a rotary plate shaker at 30 °C and 20% CO<sub>2</sub> for 24 and 48 h.

After incubation, the entire sample was transferred to a sterile microcentrifuge tube and 100 µl of 0.5% Triton X-100 was added, then the sample was mixed on a vortex mixer, using the highest setting, to produce an even suspension of spores and oil for sampling. For each 1 ml sample, three 14 µl samples were immediately sampled with a pipette and placed on a glass slide, then covered with a coverslip. Spore germination was assessed as described for the controls in the previous experiment, except that 100 spores from each drop were assessed for spore germination, for a total of 300 spores per replicate of each treatment. This sampling procedure allowed us to get a random sample of spores, despite their lipophilic nature. By adding Triton X-100 after incubation, we were able to use a higher concentration without concern of it affecting spore germination.

The experiment was set up as a random block design, as before, except that spores were collected from four bee cadavers, combined, for each block. Due to the larger number of treatments, we needed more spores than could be reliably recovered from one cadaver. Three replicate blocks were set up. ANOVA, using an arcsine-square-root transformation of the proportion germinated, was used to determine treatment effects. Duncan's LSD was used to compare treatments.

Table 1. Fatty acid composition of lipids extracted from the pollen provisions, as compared to other oils used in the experiments

Oil type	Fatty acid components (% composition) <sup>a</sup>										
	12:1	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1
Provision				38.6		2.6	1.6	17.3	39.4		
Bee prepupae <sup>b</sup>	1.2	7.8	8.2	13.9	29.5	0.6	12.9	5.0	14.8		
Canola <sup>c</sup>				4.1		1.8	63.0	20.0	8.6		1.9
Linseed <sup>c</sup>				6.0		2.5	18.0	24.1	47.4	0.5	

<sup>a</sup> The numbers to the left and right of the colon for each fatty acid are the number of carbons and the number of double bonds, respectively.

<sup>b</sup> Fatty acid composition of lipids extracted from overwintering prepupae of *M. rotundata* [11].

<sup>c</sup> From Reeves et al. [12].

### *Experiment 3: Testing mineral oil and specific fatty acids*

To determine whether the effects of lipids on spore germination were due to a physical or nutritional effect, we set up a bioassay that included mineral oil. Mineral oil is a mixture of liquid hydrocarbons refined from petroleum and does not contain FFAs or TAGs, as do biological lipids. As such, we expect it to have no nutritional value to this pathogen. In addition to testing mineral oil (a light, sterile filtrate from Sigma, St. Louis, MO), we added two FFAs to the oil to see if they would enhance germination. We selected linoleic acid (Sigma, St. Louis, MO) and palmitic acid (Sigma, St. Louis, MO) because these FFAs occurred in significant quantities in both larval bee and pollen provision extracts (Table 1), and were readily available in a stable form.

This experiment consisted of the following thirteen treatments (percents are by volume):

(1) a control where no oil was added, (2 and 3) 0.2 and 2% mineral oil, (4 and 5) 0.2 and 2% canola oil, (6 and 7) 0.2 and 2% of a 50:50 mixture of mineral oil and linoleic acid, (8 and 9) 0.2 and 2% of a 50:50 mixture of canola oil and linoleic acid, (10 and 11) 0.2 and 2% of a 50:50 mixture of mineral oil and palmitic acid, and (12 and 13) 0.2 and 2% of a 50:50 mixture of canola oil and palmitic acid.

The oils were added to the modified V-8 broth and sonicated to make an emulsion, as described above. *A. aggregata* spores were collected from bee cadavers and added to the broth, as described above. A random block design was used as described for Experiment 2, except that five cadavers were used for each replicate block, and we had three replicates. Spores were incubated in the broth using 24-well plates on a plate shaker at 30 °C, and 20% CO<sub>2</sub>. Spores were sampled for germination after 20 and 40 h of incubation, and germination was determined as described for Experiment 2.

Multivariate analysis was used to determine whether the oils and the FFAs had any effect on spore germination. The proportion of spores germinated was transformed using an arcsine of the square-root (to normalize the variances), and served as the dependent variable. First, data for all the FFA treatments were excluded, and we tested whether canola oil and mineral oil alone affected spore germination (using canola oil and mineral oil

concentrations as the main effects). We also used ANOVA to compare the effect of canola oil versus mineral oil.

Using the complete data set, we used linoleic and palmitic acid concentrations and hours of incubation as the independent variables, and the arcsine of the square-root of the proportion of spores that germinated as the dependent variable. We analyzed canola oil and mineral oil treatments independently. This allowed us to test whether each FFA had a significant effect on spore germination.

## **Results**

### *Lipid content of culture broth and pollen provisions*

V-8 broth contained only trace amounts of lipid, 3.8 nmol/ml of FFA and 17.9 nmol/ml of TAG-FA, but lipids comprised 5.7% of the dry weight of pollen provision (5.0% of the wet weight): 26.5% of the lipid was FFA, and 12.6% was TAG fatty acid. FFAs were the main components of the oils tested in the germination bioassay, and so we compared the known FFA composition of these oils with that of the pollen provision (Table 1). The pollen provision contained mostly palmitic acid (16 carbon chain:0 double bonds) and linolenic acid (18:3). The FFA composition of bee lipid extracts and the oils used in the experiment are provided in the table for comparative purposes. The FFA most prevalent in bee prepupae is palmitoleic acid (16:1), but palmitic acid (16:0), linolenic acid (18:3) and oleic acid (18:1) all occur in significant proportions [11]. Canola oil contains mostly oleic acid (18:1) [12], which was also high in the bee lipid [11], but not the pollen. Linseed oil is highest in linolenic acid (18:3) [12].

### *Lipid effects on spore germination*

#### *Experiment 1: Canola oil and bee lipids*

Both lipids significantly increased spore germination. Significantly more of the spores that attached to lipid droplets germinated, as compared to spores in the control broth (Figure 1) ( $F = 6.43$ ;  $df = 4, 31$ ;  $P \leq 0.05$ ). If we removed the control data from the analysis to compare the two lipid portions, the difference between canola oil and bee lipid extract was still not significant, but it was

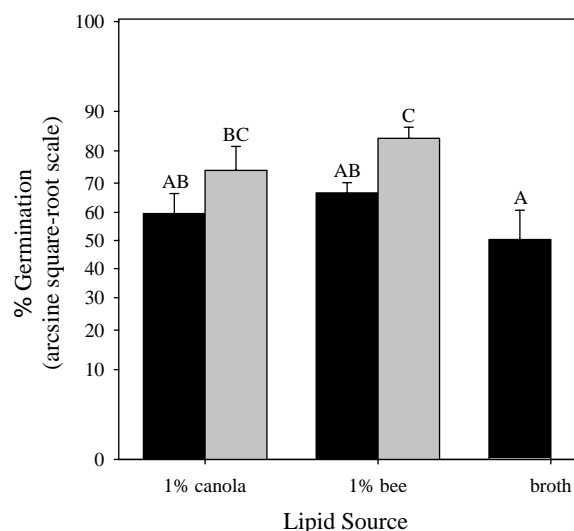


Figure 1. Effect of canola oil and bee extracts on *A. aggregata* spore germination after 24 h incubation at 30 °C and 20% CO<sub>2</sub>. Spores attached to lipid droplets (gray) and those not associated with a lipid droplet (black) were counted separately. Bars that do not have the same letter are significantly different ( $P \leq 0.05$ , Duncan's LSD,  $df = 4, 31$ ,  $F = 6.43$ ). Vertical lines above the bars represent the standard errors ( $n = 9$ ).

nearly so ( $F = 3.54$ ;  $df = 1, 24$ ;  $P = 0.072$ ). The effect of attaching to a lipid drop, however, significantly increased germination ( $F = 11.91$ ;  $df = 1, 24$ ;  $P = 0.002$ ) (Figure 1). The interactive term between lipid source and whether or not a spore was attached to a lipid droplet was not significant, and so was not included in the model. This result means that the relative effect of attaching to a lipid droplet was the same for both lipids. Variability was also affected by the treatments. The standard errors for the control broth and the canola treatment were much greater than for the bee lipid extract.

The spores attached to lipid drops in a specific orientation, with one end of the spore embedding into the lipid drop (Figure 2). The polar evagination, or swelling, typical of germinating *A. aggregata* spores occurred at the other end of the spore (Figure 2a), and the germ tube extended into the aqueous part of the broth (Figure 2b). This orientation to the lipid occurred regardless of the lipid source.

#### Experiment 2: Testing three lipids

Spore germination at 24 and 48 h did not differ significantly, so the results are only reported for

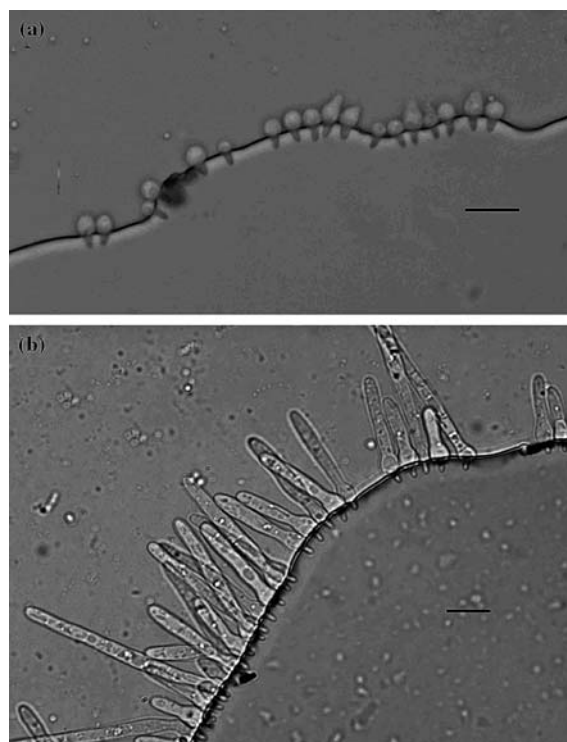


Figure 2. Spores of *A. aggregata* self-orienting along the interface between a lipid droplet (bottom portion of each image) and the aqueous portion (upper part of the images) of V-8 broth. Bars represent 10  $\mu$ m. Photos were taken at 600 $\times$  with DIC. (a) Spores during the early stages of germination, after swelling at one end into the aqueous solution. Germ tubes are just beginning to form with some of the spores. (b) Germination after 24 h of incubation, when the germ tubes are well formed. Note that one end of the spore remains in the oil and does not swell, even after germ tube formation.

24 h. Reading spore germination after 48 h of incubation was problematic because some of the hyphae had grown extensively, and thus it was difficult to determine what was once a spore and what was not.

All the lipids significantly increased spore germination (Figure 3). Increasing oil concentrations did not increase the spore germination significantly, and for linseed oil, it caused a significant decline in germination. The high concentration of linseed oil was the only treatment that was not significantly greater than the control.

#### Experiment 3: Mineral oil and FFAs

Adding oil (without added FFAs) to the broth significantly increased spore germination (Table 2), and this effect was significant for both canola oil ( $F = 16.7$ ;  $df = 1, 24$ ;  $P \leq 0.0004$ ) and

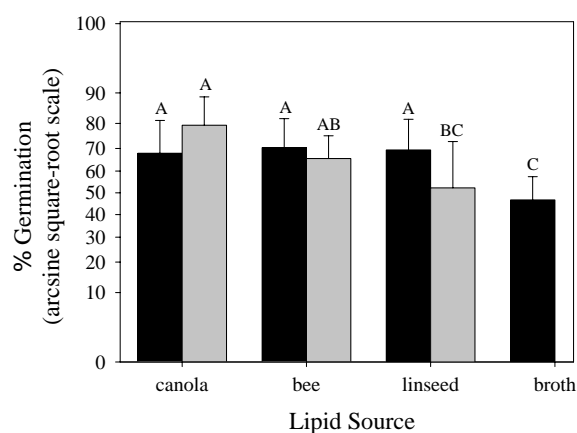


Figure 3. *A. aggregata* spore germination in the presence of lipids from different sources (24 h incubation, 30 °C, 20% CO<sub>2</sub>). Black bars are low lipid concentrations (1%, except for the broth, a control with no lipid added), gray bars are high lipid concentrations (2%). Bars that do not have the same letter are significantly different ( $P \leq 0.05$ , Duncan's LSD; df = 6, 12;  $F = 6.58$ ). Vertical lines above the bars represent the standard errors ( $n = 3$ ).

Table 2. Percent germination of *A. aggregata* spores when incubated with oils and fatty acids

Incubation period (h) <sup>a</sup>	Oil added	Total lipid conc.	Fatty acid added (50% of total lipid)		
			None	Linoleic acid	Palmitic acid
20	None	0%	48.1 (11.3) <sup>b</sup>	–	–
	Canola	0.2%	62.0 (16.8)	59.4 (9.3)	68.3 (8.4)
		2%	82.4 (5.4)	55.2 (15.8)	53.1 (26.6)
	Mineral oil	0.2%	52.9 (9.6)	61.4 (21.2)	62.0 (10.4)
		2%	82.1 (5.3)	40.7 (22.9)	74.9 (4.2)
40	None	0%	59.6 (9.5)	–	–
	Canola	0.2%	81.4 (11.7)	73.8 (8.1)	76.3 (6.0)
		2%	86.2 (4.0)	56.2 (28.0)	46.2 (25.1)
	Mineral oil	0.2%	74.4 (9.3)	60.3 (26.2)	77.0 (0.7)
		2%	85.2 (3.6)	27.8 (27.6)	84.1 (4.5)

<sup>a</sup> Spores were incubated in broth at 30 °C and 20% CO<sub>2</sub> at incubation periods of 20 and 40 h.

<sup>b</sup> Values represent mean percent germination (SE) ( $n = 3$ ).

mineral oil ( $F = 14.2$ ; df = 1, 24;  $P \leq 0.0009$ ). Germination increased significantly between 20 and 40 h incubation (in the absence of FFAs), and this effect was significant ( $F = 7.7$ ; df = 1, 24;  $P \leq 0.01$ ). Germination was significantly less with mineral oil than with canola oil ( $F = 6.9$ ; df = 2,

10;  $P \leq 0.01$ ); however, the difference was not very large (Table 2).

When FFAs were added to the oils, germination did not significantly increase between 20 and 40 h (Table 2). Adding linoleic acid to the oils decreased spore germination, and this effect was significant for mineral oil ( $F = 16.52$ ; df = 1, 30;  $P \leq 0.0003$ ), but not for canola oil (although it was nearly so,  $F = 3.4$ ; df = 1, 30;  $P \leq 0.07$ ). Linoleic acid had a toxic affect, causing the spores to lyse, and as a result it was often difficult to find spores in the high linoleic acid treatments. Palmitic acid caused a significant decrease in spore germination when mixed with canola oil ( $F = 6.8$ ; df = 1, 30;  $P \leq 0.01$ ), but not when mixed with mineral oil.

## Discussion

We found that the spores of *A. aggregata* have a polar affinity for oils, and that when they attach to oils, they are more likely to germinate. The response of the spores to oils is general in that germination rates were not much affected by the different oils tested, even though we used oils with different fatty acid compositions and mineral oil, which contains no fatty acids. Oils did not appear to be necessary for germination because approximately 50% of the spores germination in V-8 broth, even though the broth contained only minute quantities of lipids.

When alfalfa leafcutting bee larvae consume pollen provisions that are contaminated with *A. aggregata* spores, the spores germinate in the larval gut, and the germ tube penetrates the gut wall. Most fungal growth occurs in the larval body cavity [1, 2]. Spores probably do not germinate in the provision before it is eaten because the addition of hyphae to provision will not initiate infection, where as, the addition of spores will [13]. *A. aggregata* spores are known to require CO<sub>2</sub> for spore germination [7], and this requirement possibly prevents the spores from germinating in the provision before they are consumed by larvae.

We observed that *A. aggregata* spores germinated best at the interface between an oil and an aqueous material. Kish [7] described the morphology of *A. aggregata* germination, and we show here how the polar evagination that he

saw is related to the way in which the spores attached to lipid droplets. What Kish [7] calls an evagination, we think is actually a swelling at only one end of the spore, the end that is in the aqueous matter. The end of the spore that embeds in the oil does not swell at all.

It is not clear where an oil:aqueous interface might occur in the larval gut, where germination normally occurs. Further studies are needed to determine to what in the larval gut the spores are attaching. Do they attach to the peritrophic membrane or to lipid droplets released from digested pollen? Suárez-Cervera et al. [14] show that when pollen grains are digested in the gut of bee larvae, droplets of fats can form. Lipids are digested in the anterior midgut of the megachilid, *Chelostoma forisomne* [16]. If internal pollen lipids were the source of lipid for *A. aggregata* germination, then we would expect germination to occur anteriorly to the mid-gut, or in the anterior portion of the mid-gut (assuming that lipid digestion is similar for *M. rotundata*). Also, the spores might be attaching directly to pollen grains, which are surrounded by a lipid laden material called the pollenkit [17]. Dobson [17] found that most angiosperm pollens contain the majority of their FFAs in the pollenkit (for 95% of the species surveyed), and their TAGs internally (for 83% of the species surveyed). Unfortunately, alfalfa was not included in this survey. We found the alfalfa pollen provisions to be high in FFA; however, adding two of these FFAs, linoleic acid and palmitic acid, to culture broth did not increase germination, and when FFAs did have an effect on the spores, it was toxic. If lipids have nutritional value to the spores, it does not appear to be from the FFAs (although we did not test all possible fatty acids). We suspect that the effects of lipids are not nutritional so much as physical because adding mineral oil alone to the V-8 broth enhanced germination.

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*Address for correspondence:* Rosalind R. James, USDA-ARS Bee Biology and Systematics Laboratory, Department of Biology, UMC 5310, Utah State University, Logan, UT 84322-5310, USA

Phone: 435-797-0530; Fax: 435-797-0461

E-mail: rjames@biology.usu.edu